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CITATION:

Ohta, Yukari ...[et al]. Enzymatic Specific Production and Chemical Functionalization of Phenylpropanone Platform Monomers from Lignin.. ChemSusChem 2016, 10(2): 425-433

ISSUE DATE:

2016-12-16

URL:

<http://hdl.handle.net/2433/217686>

RIGHT:

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Enzymatic Specific Production and Chemical Functionalization of Phenylpropanone Platform Monomers from Lignin

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Enzymatic catalysis is an ecofriendly strategy for the production of high-value low-molecular-weight aromatic compounds from lignin. Although well-definable aromatic monomers have been obtained from synthetic lignin-model dimers, enzymatic-selective synthesis of platform monomers from natural lignin has not been accomplished. In this study, we successfully achieved highly specific synthesis of aromatic monomers with a phenylpropane structure directly from natural lignin using a cascade reaction of β -O-4-cleaving bacterial enzymes in one pot. Guaiacylhydroxypropanone (GHP) and the GHP/syringylhydroxypropanone (SHP) mixture are exclusive monomers from lignin isolated from softwood (*Cryptomeria japonica*) and

hardwood (*Eucalyptus globulus*). The intermediate products in the enzymatic reactions show the capacity to accommodate highly heterologous substrates at the substrate-binding sites of the enzymes. To demonstrate the applicability of GHP as a platform chemical for bio-based industries, we chemically generate value-added GHP derivatives for bio-based polymers. Together with these chemical conversions for the valorization of lignin-derived phenylpropanone monomers, the specific and enzymatic production of the monomers directly from natural lignin is expected to provide a new stream in "white biotechnology" for sustainable biorefineries.

Introduction

Biorefineries for obtaining sustainable energy and chemical production from renewable biomass have been the focus of in-

tensive research and development owing to the depletion of world petroleum reserves together with global warming due to anthropogenic greenhouse gas emissions.^[1] In particular, lignocellulosic biomass such as woody feedstock, agricultural waste, and perennial grass can be used as raw materials because they are abundant and not used for food and feed production.^[2–4]

The main constituents of lignocellulosic biomasses are cellulose, hemicellulose, and lignin.^[5] Processes for converting cellulose and hemicellulose to bioethanol and chemicals have been developed, and they play a central role in commercial practices worldwide.^[4,6] Although lignin is considered to be the major renewable source of high-value aromatic compounds because of its intrinsic polyaromatic chemical structure, its use has been mostly limited to low-value applications such as solid fuels and admixtures for concrete.^[7,8] Many efforts have been made to develop thermochemical, catalytic, and enzymatic strategies for the efficient production of high-value low-molecular-weight aromatic compounds from lignin.^[8–12] Although substantial yields have been achieved using synthetic lignin-model compounds, the yield and product distribution obtained from natural lignin depend highly on the specific lignin structure, which is often modified and repolymerized in complex ways during preparation.^[13] The products of this process are almost always highly heterogeneous and thereby hinder lignin valorization.^[14]

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Supporting Information for this article can be found under:
<http://dx.doi.org/10.1002/cssc.201601235>.

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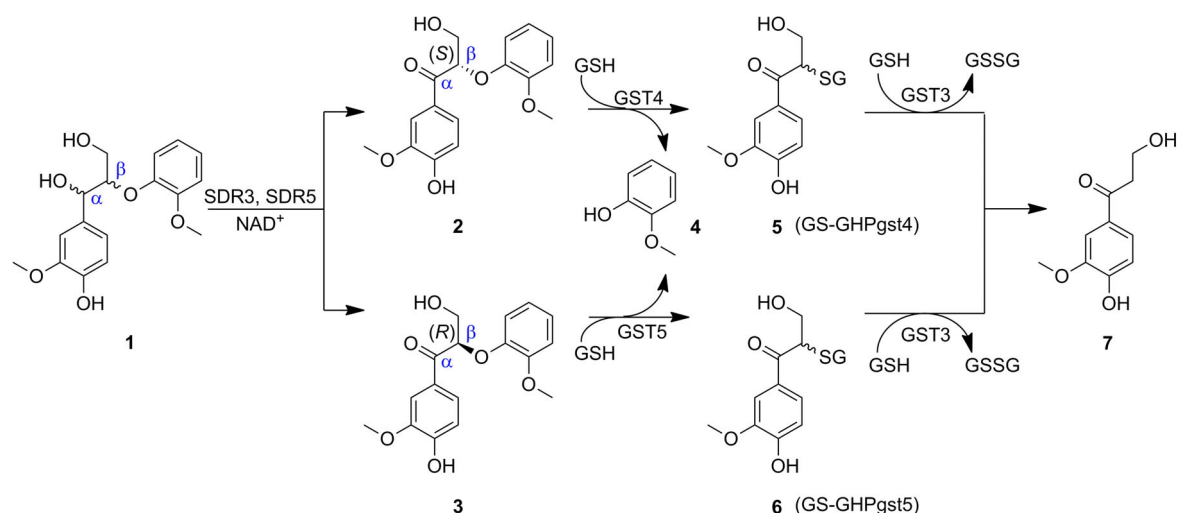
For example, Lancefield et al.^[15] reported an isolation method for simple aromatic monomers with a phenylpropanone structure via selective oxidation of benzylic alcohol at the C α position, followed by reductive cleavage of the β -O-4 linkages using zinc as the reductant. Rahimi et al.^[16] reported that the formic-acid-induced preoxidized lignin depolymerization produced aromatic monomers with structural variations, including diketones, aldehydes, and carboxylic acids. Although these methods offer some technological applications in definable aromatics production, they require toxic compounds, heavy metals, and high-temperature reactions. Thus, more environmentally friendly methods need to be developed.

An alternative for the specific production of aromatic monomers from lignin is enzymatic catalysis. Several enzymes can catalyze the selective cleavage of β -O-4 linkages in lignin-model dimers.^[17] Aromatic monomers were produced from lignin-model synthetic dimers via a cascade reaction comprising three steps, which involved the use of multiple enzymes derived from the *Sphingobium* sp. strain SYK-6. The cascade required six reactions for the conversion of racemic lignin-model dimers with two chiral carbons at the α and β positions into respective monomers. In addition to the enzymes from the strain SYK-6, a homology-based amino-acid database search for β -O-4-cleaving enzymes were identified from two *Novosphingobium* genomes.^[18,19] All six enzymatic reactions in the cascade were strictly stereospecific.^[20–22] Prior to our study, no enzyme capable of efficiently reacting with the β (S)-isomer in the last step has been identified. Owing to the lack of genetic information regarding the enzyme responsible for the conversion of the β (S)-intermediates into aromatic monomers, a recombinant enzymatic process capable of producing aromatic monomers could not be developed.^[23] Currently, aromatic monomer production from lignin preparations using enzymes has

not been successful and has resulted in only a trace amount of aromatics, such as ferulic acid and vanillin from alkali lignin.^[23]

Recently, we reported that a combination of six enzymes produced using genes from a marine *Novosphingobium* strain, which was isolated from sunken wood in Suruga Bay, Japan,^[24] entirely converted a racemic lignin-model dimer into its respective monomer in three steps (Scheme 1).^[17,25] Two short-chain dehydrogenase/reductases (SDRs; EC 1.1.1.–) (SDR3, SDR5) and two glutathione S-transferases (GSTs; EC 2.5.1.18) with β -etherase activity (GST4, GST5) that catalyze the first and second steps, respectively, were strictly stereospecific. Surprisingly, GST3 catalyzed the third step by efficiently removing glutathione from both compounds **5** and **6** (Scheme 1), which were produced from a racemic lignin-model dimer (Scheme 1 a mixture of compounds **2** and **3**). The discovery of the non-stereoselective enzyme GST3 paved the way to convert all stereoisomers of compound **1** to their respective monomers for the first time. However, a feasible method of processing natural lignin of biomass origins using these five enzymes remains unknown in terms of reactivity and selectivity for highly heterogeneous natural lignin substructures.

In this study, we investigated the optimal reaction conditions for GST3. Using the five enzymes shown in Scheme 1, we enzymatically produced phenylpropanone monomers from isolated natural lignin. To elucidate the catalytic mechanism of the β -O-4 cleavage of natural lignin, we analyzed the intermediates produced from lignin. Finally, we assessed the future prospects of these phenylpropanone monomers as new platform chemicals derived from natural lignin.



Scheme 1. Enzymatic cascade for GHP (compound **7**) synthesis from lignin-model dimers (GGGE, compound **1**) via MPPHV (compound **2**, **3**). The responsible enzymes and their required cofactors are shown. Abbreviations: SDR—short-chain dehydrogenase–reductase, GST—glutathione S-transferase; GS-GHPgst4 (compound **5**)—glutathione conjugate of GHP produced by β -O-4 bond cleavage and removal of guaiacol (compound **4**) by GST4; GS-GHPgst5 (compound **6**)—glutathione conjugate of GHP produced by GST5; NAD⁺—oxidized form of nicotinamide adenine dinucleotide (NAD); GSH—reduced form of glutathione; and GSSG—oxidized form of glutathione. Protein accessions in the DDBJ/EMBL/GenBank database: SDR3 (GAM05523), SDR5 (GAM05547), GST3 (GAM05529), GST4 (GAM05530), and GST5 (GAM05531).

Results and Discussion

Optimal pH and temperature for GST3 activity

We identified the optimal pH values and temperatures for the activity and kinetic parameters of SDRs (SDR3, SDR5) and GSTs (GST4, GST5).^[25] In contrast, GST3 characterization has not been directly performed because the substrates for GST3, glutathione-conjugated guaiacylhydroxypropanones (GS-GHPs) (Scheme 1, compounds 5 and 6), have not been commercially available. Here, we enzymatically produced GS-GHPs from racemic 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1-propanone [(2-methoxyphenoxy)hydroxypropiovanillone; MPHPV] (Scheme 1, compounds 2 and 3) using GST4 and GST5. The reaction products were designated as GS-GHPgst4 and GS-GHPgst5 (Scheme 1, compounds 5 and 6), respectively. Then, we purified each of the GS-GHPs. Using purified each of the GS-GHPs as a substrate, we investigated the effect of pH value on GST3 activity. The optimum pH value and temperatures for GST3 activity were approximately 8.0 (Figure 1a, b) and 25–30 °C (Figure 1c, d), respectively, both for GS-GHPgst4 and GS-GHPgst5. The specific activities in 0.1 M *N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid buffer (TAPS) at pH 8.5 and 15 °C were 3.9 and 9.1 U mg⁻¹ protein for GS-GHPgst4 and GS-GHPgst5, respectively.

GHP (Scheme 1, compound 7) synthesis via enzymatic cascade reactions from milled wood lignins

Each enzyme retained more than 80 % of the respective maximal activity at pH 8.5.^[25] The optimal temperature of SDR3 was 15 °C, lower than those of the other enzymes, indicating the instability of SDR3 at a higher temperature. SDR3 was thought to be the limiting reaction step in the enzymatic cascade reactions. Accordingly, the following enzyme reactions were conducted under optimal conditions for SDR3 activity (0.1 M TAPS at pH 8.5 and 15 °C) using the enzymes simultaneously in one pot.

Milled wood lignins (MWLs) from softwood and hardwood were prepared from Japanese cedar (*Cryptomeria japonica*) and *Eucalyptus globulus* wood and are here designated C-MWL and E-MWL, respectively. The main product from C-MWL was determined to be GHP according to the retention time (*t_R*) on the liquid chromatography–mass spectrometry (LC–MS) chromatograph, observed molecular mass, calculated elemental composition, fragmentation pattern on mass spectra, and UV spectrum using authentic GHP as a reference (Figure 2a, c and Figure S5a, c in the Supporting Information). The yield of GHP was 2.4 ± 0.005 wt % [24 ± 0.05 mg g_{lignin}⁻¹]. The main products from E-MWL were determined to be GHP and syringylhydroxypropanone (SHP) using authentic GHP and SHP as references

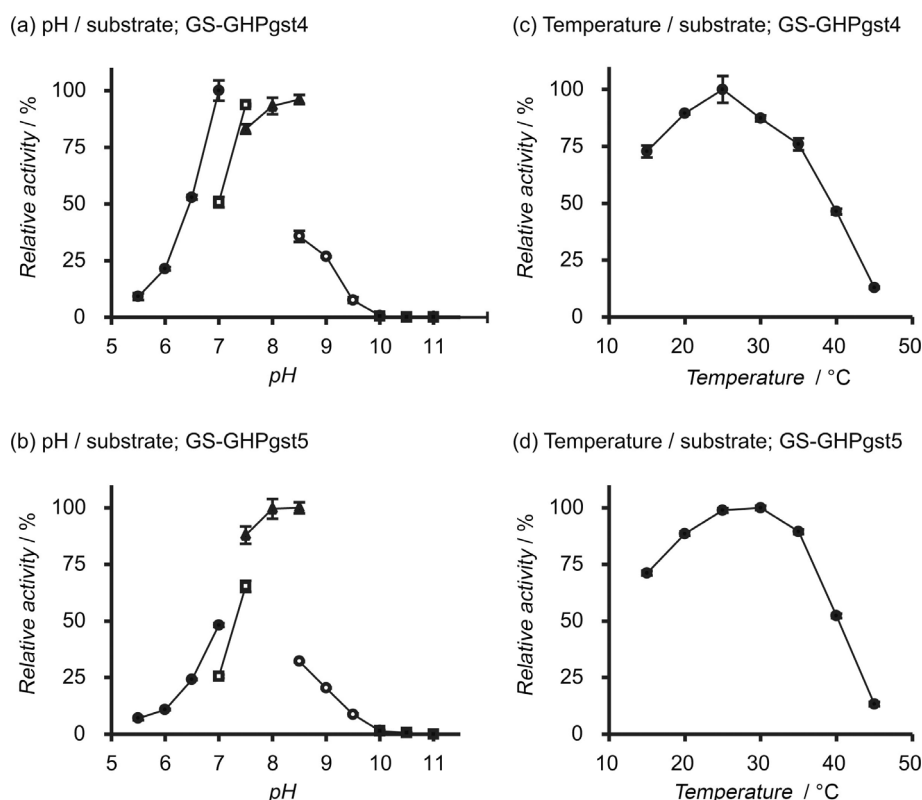


Figure 1. pH–activity curves of the purified GST3 using (a) GS-GHPgst4 and (b) GS-GHPgst5 as the substrates. The buffers used were 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES; pH 5.5–7.0; ●), 3-[*N*-morpholino]propanesulfonic acid (MOPS; pH 7.0–8.0; □), TAPS (pH 8.0–9.0; ▲), *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES; pH 9.0–10.0; ○), *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS; pH 10.0–11.0; ■). The activity was measured in the buffers including 25 °C. The values are shown as percentages of the maximal activity of GST3 observed at pH 7 for GS-GHPgst4 and pH 8 for GS-GHPgst5, which are taken as 100%. The temperature–activity curves of the purified GST3 using (c) GS-GHPgst4 and (d) GS-GHPgst5 as the substrates are also shown. The values are shown as percentages of the maximal activity of GST3 observed at 25 °C for GS-GHPgst4 and 30 °C for GS-GHPgst5, which are taken as 100%.

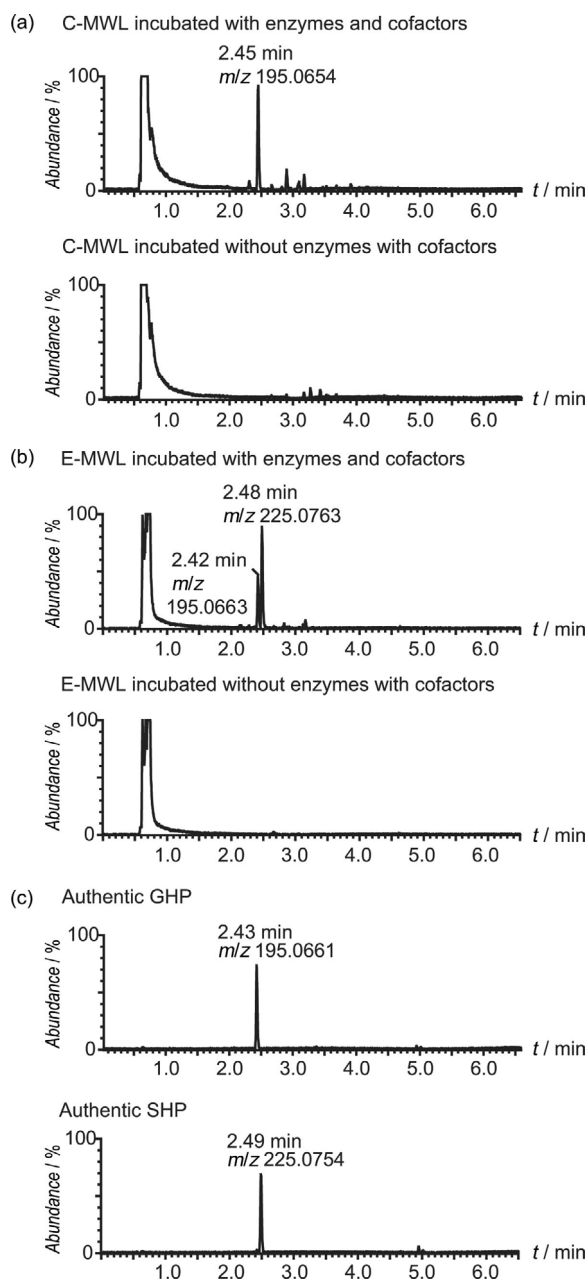


Figure 2. One-pot enzymatic production of GHP and SHP from (a) C-MWL and (b) E-MWL. Total ion chromatograms obtained from LC-MS analysis of the reaction of five enzymes (SDR3, SDR5, and GST3-5) with the MWLs and cofactors are shown (top). The reactions were conducted under the same conditions without enzymes but with cofactors to assess the non-enzymatic production of GHP/SHP (bottom). (c) Authentic GHP (top) and SHP (bottom) analyzed under the same conditions.

(Figure 2b, c and Figure S5b, c in the Supporting Information). The yields of the two compounds were 1.9 ± 0.012 and 4.7 ± 0.025 wt% [19 ± 0.12 and 47 ± 0.25 mg g_{lignin}⁻¹], respectively. To the best of our knowledge, this is the first report of enzymatic production of phenylpropanone monomers from MWL.

There have been numerous studies on the production of aromatic monomers from lignin.^[7–9] For example, pyrolysis and cracking processes have been extensively studied for decades. The major products from different types of pyrolysis processes

are complex mixtures of deoxygenated aromatic monomers such as vinylphenols, toluene, xylene, and many other aromatics along with gaseous products, that is, hydrocarbons of low molecular weight. For the cracking process, a high yield of approximately 80 wt% was recorded for a mono-/oligomeric phenolic mixture. The common drawback of pyrolysis and cracking processes is the required harsh operating conditions (a high temperature ranging between 300–650 °C under high pressures) and side reactions that are difficult to control. To overcome these problems, hydrogenolysis and hydrolysis under subcritical/supercritical conditions with various catalysts operating at temperatures below 300 °C have been attempted and have drawn attention for their potential to be viable new methods.^[9] As an example of a significant study in this field, nickel-based catalysts were used for the selective production of propylguaiacol and propylsyringol from sawdust with approximately 50 wt% of lignin conversion at 200 °C.^[26] The supply of hydrogen and recycling of the catalysts is a major issue in the process that has to be addressed. Another excellent contribution in chemical catalysis was reported by Lancefield et al.^[15] The production of the phenolic monomers (GHP and SHP) was conducted at 80 °C using a chlorinated oxidizer and zinc as a reductant under atmospheric pressure in the presence of air. The production yields of GHP and SHP from a birch lignin preparation were 0.46 and 4.6 wt%, respectively. Although these values depend strongly on the biomass source and process of lignin preparation,^[13] the yields of enzymatically produced GHP and SHP in this study were comparable to those obtained by chemical catalysis using toxic halogenated aromatics and heavy metals. The theoretical yield of these monomers (GHP plus SHP) was calculated to be approximately 12 wt% based on the content of releasable β-O-4 linkages.^[15] The experimental yields obtained by Lancefield et al. and by us reported herein were similar and approximately half of the theoretical value. The yield limitation in the chemical catalytic process was estimated to be owed to the formation of by-products that include unknown repolymerized products. We believe that in our ongoing and future studies it should be possible to increase the yields obtained in our study by improving the catalytic property of the enzymes, for example, by improving their catalytic efficiencies and substrate recognition capacities as well as improving their protein stabilities through protein engineering. Furthermore, conducting the enzymatic process under mild conditions shows promise for avoiding repolymerization, which is likely the cause for the formation of undesired by-products.

Enzymatic production of aromatic monomers is an attractive alternative to thermal and chemical conversion of lignin. Many microbial enzymes have been reported to have the ability to produce lignin-related aromatic monomers. Although fungal and bacterial peroxidases can break the linkages of inter lignin monomers using extracellular radical molecules, the enzyme activity and specificity is low and can even cause repolymerization during the enzymatic reaction. Other bacterial enzymes have been examined to produce aromatic monomers including vanillin, caffeic acid 4-vinylguaiacol, coniferyl alcohol, and GHP using ferulic acid,^[27] eugenol,^[28] and lignin-model dimers^[22,23]

as starting materials; however, these enzymes have not successfully been used for natural lignin preparations. In contrast, there have been a few reports demonstrating microbial production of aromatic monomer directly from lignin. For example, metabolically engineered *Rhodococcus jostii* RHA1 reportedly produced vanillin up to 96 mg mL⁻¹ after a 6-day cultivation in the media containing lignocellulose.^[29] Although this study demonstrated the possibility of biological production of the aromatic monomer, vanillin is already produced chemically from lagnosulfonate at industrial scale.^[9] In our study, the enzyme reactions were conducted with MWLs suspended in an aqueous buffer with a small amount of organic solvent. At present, we use *N,N*-dimethylformamide to dissolve MWL. However, in a scaled-up production we propose that a safer and greener solvent should be used instead. Biomass-based solvents with lower boiling points than the products, such as acetone, butanol, or tetrahydrofuran,^[30] are potential candidates to increase the sustainability of the enzymatic production of GHP and SHP.

In general, the productivity must reach 1–3 g L⁻¹ h⁻¹ for the products to reach economic viability for bio-based chemicals.^[31] To meet this goal, the productivity (approximately 5 mg L⁻¹ h⁻¹ of the products in the current experimental setting) must be increased by 2–3 orders of magnitude by operating at higher volumetric concentrations. In addition, it is necessary to enhance the enzymatic activity and stability by protein engineering and stoichiometric optimization of the multiple reactions^[32] to reduce the loadings on the enzymes and improve the yields. In addition, recycling of the cofactors of NAD⁺ and GSH as well as an eco-friendly pretreatment for biomasses suitable for enzymatic reactions are needed for the cascade presented in this study to become economically sound.

Product recovery was conducted in this study by liquid-phase extraction using ethyl acetate as an organic solvent. The other practical product recovery options need to be assessed and optimized because the product recovery processes accounts for a major part of a total cost and is critical for achieving sustainable production of bio-based chemicals.^[33] In addition, the residual insoluble and soluble lignin fractions have to be recovered and analyzed for efficient utilization for entire lignin valorization. Although there are many problems to overcome, further optimization of each process in the upstream and downstream processes are possible and provide grounds for further study.

Analysis of substrate recognition tolerance of the enzymes for MWL substructures

The bacterial enzymes involved in the cleavage of β -O-4 ether linkages have been considered to show substantial activity only on dimeric lignin-model synthetic compounds, with low activity on polymeric substrates.^[18] However, our enzyme system produced phenylpropanone monomers. To elucidate the reaction mechanism of the enzymes displaying substrate recognition tolerance for unknown substructures present in MWL, we analyzed the reaction products from C- and E-MWL by the four enzymes (SDR3, SDR5, and GST4-5). The gluta-

thione conjugates produced by the enzymes were analyzed by LC-MS (Figure S6a, b in the Supporting Information). The glutathione conjugates obtained from authentic guaiacylglycerol- β -guaiacyl ether (GGGE, Scheme 1, compound 1) by the same enzyme set were analyzed under the same conditions as the references (Figure S6c in the Supporting Information).

The observed molecular masses in the LC-MS analyses and calculated elemental compositions of the main two reaction products (t_R ; 1.69 and 1.97 min) from C-MWL were the same, m/z 500.13 (Figure S7a in the Supporting Information) and C₂₀H₂₆N₃O₁₀S, in agreement with those of the deprotonated parent molecular ion of glutathione-conjugated GHP [GS (elemental composition, C₁₀H₁₆N₃O₆S)-C₁₀H₁₁O₄]. The t_R on LC-MS chromatograms, observed molecular mass, and fragmentation patterns on the mass spectra of the major products from C-MWL agreed well with those of the products from the enzyme reactions using GGGE as a substrate (Figure S7c in the Supporting Information). From these results, the two peaks were inferred to be isomers. Four major products were produced in the case of E-MWL (Figure S8b in the Supporting Information). MS data from two peaks (t_R ; 1.64 and 1.96 min) were the same as those from C-MWL, and the others (t_R ; 1.86 and 2.03 min) were molecules with m/z 530.14 and calculated compositions of C₂₁H₂₈N₃O₁₁S, in agreement with those of the deprotonated parent ion of glutathione-conjugated SHP (GS-C₁₁H₁₃O₅). The MS spectra contained several fragment ions, such as m/z 272.09 and 254.08 (Figure S8a–c in the Supporting Information), in common with the MS spectrum obtained from authentic glutathione (Figure S7d in the Supporting Information). These fragment ions were speculated to have been derived from the conjugated glutathione.

LC-MS/MS analyses revealed that the reactions using C- and E-MWL as the substrates produced numerous molecules with different molecular size, providing deprotonated molecular ions ranging from m/z 500.13 to 964.30 (Figure S8 in the Supporting Information). The observed molecular masses, fragment ions, and calculated elemental compositions are listed in Table 1. These results suggested that these enzymes could accommodate not only lignin-model dimers but also lignin oligomers consisting of approximately 10–30 carbon atoms as their substrates and cleave the β -O-4 linkages in natural lignin with highly heterologous substructures.

GHP transformation into various derivatives

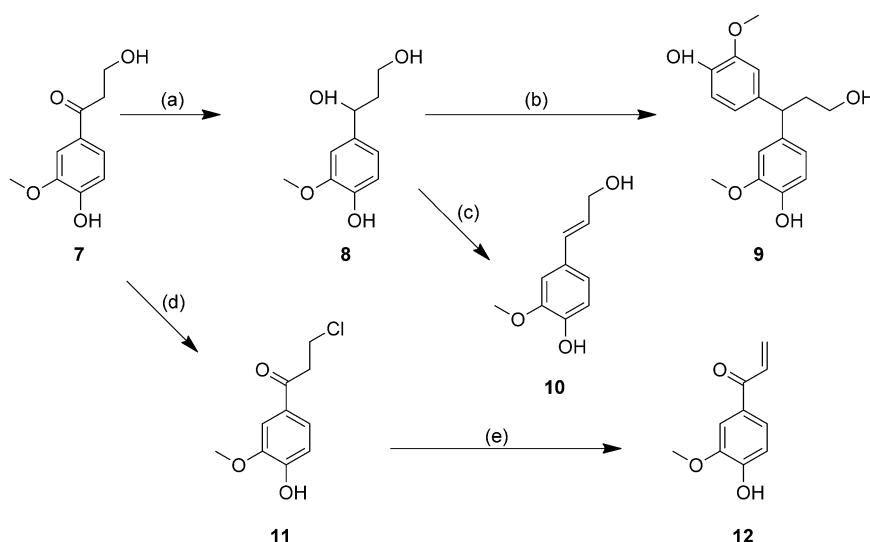
We investigated the chemical conversion of GHP to show the feasibility of effective lignin use with a wide range of applications (Scheme 2).

The simple and convenient 1-guaiacyl-1,3-propanediol (GPD, 1-(4-hydroxy-3-methoxyphenyl)-1,3-propanediol, Scheme 2, compound 8) synthesis from GHP was achieved by the reaction of GHP with NaBH₄ (Scheme 2). The reaction proceeded completely at room temperature in aqueous NaOH to afford GPD in 79%. The pH adjustment to approximately 6–9 was important for the efficient separation of GPD from the reaction mixture. Some methods for synthesizing GPD have been reported, but multi-step processes are required.^[34, 35]

Table 1. Glutathione-conjugated intermediates obtained from MWLs produced by enzymatic reaction (SDR3, SDR5, GST4, and GST5) detected by LC-MS/MS.

Detected parent ion [M-H] [−] ; [HRMS, <i>m/z</i>] ^[a]	Source	Retention time ^[b] [min]	Detected fragment ion ^[c] [<i>m/z</i>]	Calculated elemental composition ^[d]
500.1342	C-MWL	1.59, 1.94	482, 470, 464, <u>272</u> , <u>254</u> , <u>210</u> , <u>179</u> , <u>143</u> , <u>128</u>	C ₂₀ H ₂₇ N ₃ O ₁₀ S [GS-C ₁₀ H ₁₁ O ₄]
500.1318	E-MWL	1.52, 1.93	482, 470, 464, <u>272</u> , <u>254</u> , <u>210</u> , <u>179</u> , <u>143</u> , <u>128</u>	C ₂₀ H ₂₇ N ₃ O ₁₀ S [GS-C ₁₀ H ₁₁ O ₄]
530.1464	E-MWL	1.95	512, 500, 494, 383, <u>272</u> , <u>254</u> , 239, <u>210</u> , <u>179</u> , <u>143</u> , <u>128</u>	C ₂₁ H ₂₉ N ₃ O ₁₁ S [GS-C ₁₁ H ₁₃ O ₅]
636.1857	C-MWL	2.31	363, <u>306</u> , <u>288</u> , <u>272</u> , 137	ND
678.1962	C-MWL	2.58	660, 648, 642, <u>272</u>	C ₃₀ H ₃₇ N ₃ O ₁₃ S [GS-C ₂₀ H ₂₁ O ₇]
678.1972	E-MWL	2.57	660, 648, <u>272</u>	ND
706.1913	E-MWL	2.51	688, 419, <u>272</u> , <u>254</u>	ND
738.2160	E-MWL	2.43	720, <u>306</u> , <u>272</u> , <u>254</u>	ND
856.2609	C-MWL	2.92	855, 838, <u>306</u>	ND
874.2701	C-MWL	2.56	856, <u>306</u> , <u>272</u>	C ₄₀ H ₄₉ N ₃ O ₁₇ S [GS-C ₃₀ H ₃₃ O ₁₁]
874.2723	E-MWL	2.56	856, 843, 838, 802, 782, 519, 466, 321, <u>272</u> , 207, <u>143</u> , 140	C ₄₀ H ₄₉ N ₃ O ₁₇ S [GS-C ₃₀ H ₃₃ O ₁₁]
932.3058	E-MWL	3.06	<u>306</u> , 305, <u>272</u>	ND
964.3091	E-MWL	2.55	963, 946, 782, 536, <u>306</u> , <u>272</u>	ND

[a] HRMS: high-resolution MS. [b] The MS chromatograms obtained from LC-MS/MS analysis after enzymatic reactions with C- and E-MWLs are shown in Figure S9 in the Supporting Information. [c] The fragment ions derived from glutathione (Figure S8d in the Supporting Information) are underlined. [d] ND: not determined; GS: conjugated glutathione (elemental composition: C₁₀H₁₆N₃O₆S[−]).



Scheme 2. Chemical synthesis that yields functional chemicals from GHP (7), a key platform chemical. Reaction conditions: (a) NaBH₄, 26 °C, 24 h, 79% for GPD (compound 8); (b) methanesulfonic acid, 65 °C, 22.5 h, 49% for BGP (compound 9); (c) isopropanol and trimethylamine, 85–90 °C, 48 h, 79% for coniferyl alcohol (compound 10); (d) HCl, 90 °C, 96% for 3-chloro-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (compound 11); and (e) sodium ethoxide, RT, 1.5 h, 61% for GVK (compound 12).

3,3-Bisguaiacyl-1-propanol (BGP, 3,3-bis(4-hydroxy-3-methoxyphenyl)-1-propanol, Scheme 2, compound 9) was synthesized from GPD under acidic conditions. We found that GPD shows high reactivity with both acids and alkalis. In the presence of an acid catalyst, its reactivity leads to the formation of BGP (Scheme 2b, Figure S2a,b in the Supporting Information), a bisphenol that may be used as a raw material for epoxy resins; as a hardener for epoxy and urethane resins; and as a raw material for new functional polyesters and polycarbonates. In the earlier study,^[36] the reaction of GPD with an excess of phenol was performed using hydrochloric acid to give 3-(hydroxy-

phenyl)-3-(4-hydroxy-3-methoxyphenyl)-1-propanol, an unsymmetry compound and possibly a mixture, in view of the two reaction sites of phenol.

Coniferyl alcohol (Scheme 2, compound 10) synthesis from GPD under basic conditions. Coniferyl alcohol is a monolignol, which is a precursor of lignin biosynthesis,^[37] and has important applications in research areas as an artificial lignin. Coniferyl alcohol can be converted to valuable aromatic compounds, such as medicines for cancers and diabetes; functional foods and cosmetics with antioxidizing activity; and pesticides.^[38] We achieved the formation of coniferyl alcohol from

GPD under heating conditions using triethylamine as the base and isopropanol as the solvent (Scheme 2c). The choice of base and solvent was crucial because coniferyl alcohol tends to polymerize under basic conditions, particularly in aqueous solvents. The yield was higher with an organic base such as triethylamine. Methanol or ethanol as the solvent led to the formation of 3-(4-hydroxy-3-methoxyphenyl)-3-alkoxy-1-propanol (an alkoxylated compound) as the main product.

Guaiacylvinylketone (GVK, 1-(4-hydroxy-3-methoxyphenyl)-2-propene-1-one, Scheme 2, compound 12) synthesis was performed from GHP according to a method used for 1-(4-hydroxyphenyl)-2-methyl-2-propene-1-one synthesis from 1-(4-hydroxyphenyl)-3-hydroxy-2-methyl-1-propanone.^[39] The reaction of GHP with hydrochloric acid was conducted at approximately 70 °C to provide a high yield of 3-chloro-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (96%)(Scheme 2d, compound 11). The chlorinated product was treated with sodium ethoxide in ethanol to GVK in 61 % (Scheme 2e, compound 12). We found that this dehydrochlorination reaction also proceeded in NaOH aqueous solution under mild conditions, between room temperature and approximately 60 °C. In our preliminary polymerization experiment of GVK using azobisisobutyronitrile and tetrahydrofuran as the radical initiator and solvent, respectively, a solid was obtained. The ¹H-NMR spectrum of the compound confirmed the presence of aliphatic methylene and methine protons and the absence of vinyl protons (Figure S4 in the Supporting Information), indicating polymerization via the vinyl group.

Conclusions

An enzymatic strategy for the highly selective production of aromatic monomers with phenylpropanone structure from MWL isolated from hardwood and softwood was demonstrated using five enzymes in one pot. The recognition capacity of the broad substrate range by these enzymes may shed light on the mechanism by which the enzymes cleave β-O-4 linkages in natural lignin preparations that show high structural heterogeneity. The selectivity of the enzymatic one-pot reaction under mild conditions is appealing. Still, the process presents problems to overcome, including the costs of the enzyme and cofactor production, wastewater treatment, and utilization of the remaining lignin fraction. All the derivatives obtained in this study retain the aromatic rings, phenolic hydroxyl groups, and methoxy groups in their structure. This finding suggests that these compounds have various applications as lignin-derived materials. The chemical conversions in this study demonstrated that the enzymatically-produced phenylpropanone monomers will be the platform aromatic chemicals for sustainable industries based on woody biomasses.

Experimental Section

General methods

Commercially available compounds were purchased and used as received unless otherwise stated. ¹H and ¹³C NMR spectra were re-

corded on a Varian Inova 400- and 500-MHz spectrometer. Multiplicities were described using the following abbreviations: s, singlet; d, doublet; t, triplet; and m, multiplet; the J couplings were reported in Hz. IR spectra were obtained on a JASCO FT/IR-6200 type A Fourier transform IR spectrophotometer from KBr discs; only characteristic peaks were reported. LC–MS data were generated using a Waters Xevo G2 quadrupole time-of-flight mass spectrometer operated in negative ion ESI mode. The inlet system was a Waters Acquity H-class UPLC system and was operated at a flow rate of 0.4 mL min⁻¹ using a BEH C18 reverse-phase column (1.8 μm particle size, 100×2.1 mm; Waters) using the mobile-phase gradients A (2-mM sodium acetate and 0.05 % formic acid) and B (95 % acetonitrile/H₂O) under the following conditions: 0–6 min, 95%–5 % A with B as the remaining eluent; and from 6–7 min, 100 % B. The eluate was monitored at 270 nm using a Waters photo diode array (PDA) eλ detector. Data were acquired over the mass range of 100–1000 Da with a 0.45 s scan time using a desolvation temperature of 500 °C, source temperature of 150 °C, and cone voltage of 30 V. HPLC analysis was performed using a Waters Alliance 2796 liquid chromatography (LC) system equipped with an Xbridge C18 reversed-phase column (3.5 μm particle size, 100×4.6 mm; Waters) operated at a flow rate of 1.2 mL min⁻¹ using the mobile-phase gradients A (2 mM sodium acetate and 0.05 % formic acid) and C (95 % methanol/H₂O) under the following conditions: 0–1 min, 90 % A and 10 % C; 1–8 min, a decreasing gradient of 90%–10 % A with C as the remaining eluent; followed by 8–10 min 100 % C. The eluate was monitored at 270 nm using a Waters 2998 PDA detector. Chiral chromatography of the enzymatic reaction mixtures was performed using an Alliance 2796 LC system equipped with a CHIRALPAK IE-3 column (4.6×250 mm; Daicel Chemical Industries). A acetonitrile and H₂O mixture was used as the mobile phase at a flow rate of 1.0 mL min⁻¹. The acetonitrile concentration of the mobile phase was adjusted as follows (the remaining eluent was H₂O): 0–10 min, 20 % acetonitrile; 10–15 min, gradient from 20 % to 30 % acetonitrile; and 15–30 min, 30 % acetonitrile. The eluate absorbance was monitored at 270 nm using a Waters 2998 PDA detector. The recombinant enzymes [SDR3 (accession; GAM05523), SDR5 (accession; GAM05547), GST3 (accession; GAM05529), GST4 (accession; GAM05530), and GST5 (accession; GAM05531)] were prepared as previously reported.^[25]

Lignin-model dimer synthesis

GGGE (Scheme 1, compound 1) and MPHPV (Scheme 1, compound 2, 3) were synthesized as previously described.^[25]

Biochemical characterization of GST3

A glutathione-conjugated intermediate (GS-GHP) was enzymatically produced by the reaction of either GST4 and GST5 with 2-mM MPHPV using 4-mM glutathione as a cofactor at 20 °C for 16 h. Each of the products (GS-GHPgst4 and GS-GHPgst5) was then purified by solid-phase extraction from the respective reaction mixture, as follows. The reaction mixture was diluted 10-fold with methanol and applied to a phenomenex Strata extra clean NH₂ column. After the column was washed with distilled water, GS-GHP was eluted with 10-mM NaCl. GS-GHP concentration was calculated based on GHP concentration determined by HPLC after complete removal of conjugated glutathione by addition of excess GST3 (0.2 mg mL⁻¹) and incubation at 20 °C for 16 h. GST3 was reacted with 1 mM GS-GHPgst4 or GS-GHPgst5 as a substrate and 2 mM glutathione as a cofactor at 25 °C for 30 min. The formation of the reaction product GHP was measured by HPLC. The determination of the pH opti-

mum for GST3 activity was performed using the following buffers (0.1 M): MES (pH 5.5–7.0), MOPS (pH 7.0–8.0), TAPS (pH 8.0–9.0), CHES (pH 9.0–10.0), and CAPS (pH 10.0–11.0). The optimal temperature was determined by quantifying the reaction product (GHP) after a 30 min reaction in 0.1 M TAPS, pH 8.5. All experiments were performed in triplicate.

MWL preparation and enzyme reaction with MWLs

The wood meal was extracted with a toluene and ethanol (2:1, v/v) mixture using a Soxhlet extractor for 10 h. The wood meal was dried at 105 °C for 12 h and finely divided in a vibratory ball mill for 48 h under a nitrogen atmosphere with constant cooling using running water. The wood meal was extracted with 96% aq. dioxane for 24 h at room temperature. The extract was evaporated and then freeze-dried. The crude MWL was dissolved in 90% aq. acetic acid and then precipitated from distilled water. The precipitates were dissolved in a 1,2-dichloroethane and ethanol (2:1, v/v) mixture and added to diethyl ether. The precipitate was washed with petroleum ether and evaporated to give the MWL fractions. C- or E-MWL was dissolved at 10% w/v in *N,N*-dimethylformamide and then suspended at 0.2% w/v in 0.1-M TAPS, pH 8.5. The mixture was incubated with enzymes (SDRs, 50 mU mL⁻¹ and GSTs, 5 mU mL⁻¹) at 15 °C for 24 h in the presence of cofactors (10 mM NAD sodium salt and 20 mM glutathione). Parallel reactions were conducted without enzymes to assess the non-enzymatic production of GHP/SHP. For quantification of the reaction products, the reaction mixtures were extracted three times with four volumes of ethyl acetate. The extract was dried under N₂ gas at room temperature and then dissolved in 20% acetonitrile and analyzed with LC-MS coupled with a UV detector. The GHP/SHP concentrations in samples were calculated from the peak area under the UV chromatogram at 270 nm using authentic GHP/SHP as standards. All samples were normalized to ethyl ferulate as an internal standard. All reactions were performed in triplicate.

Chemical synthesis of GHP derivatives

GHP (2.05 g, 10.5 mmol) was dissolved in a 200-mL Erlenmeyer flask containing 100 mL water and 1-N aqueous sodium hydroxide (11.2 g, 11.2 mmol). Sodium borohydride (0.48 g, 12.7 mmol) was slowly added over approximately 10 min at room temperature (26 °C); the mixture was allowed to react for 24 h at 26 °C. After confirming the complete conversion of GHP by HPLC, 10% aqueous hydrochloric acid (6.1 g) was slowly added dropwise to adjust the pH to 9.0. The mixture was extracted twice with 70 mL of 1-butanol and then the combined 1-butanol layers were washed with 30 mL of 1.5% aqueous sodium bicarbonate and further with 30 mL of water. 1-butanol was removed by reduced-pressure distillation to provide 1.63 g of transparent, viscous GPD in a 79% yield. The ¹H-NMR spectrum (Figure S1 in the Supporting Information) and HRMS of synthetic GPD were as follows.
¹H-NMR (D₂O): δ = 1.93–2.12 (m, 2H, –CH₂–), 3.57–3.70 (m, 2H, –CH₂–O–), 3.90 (s, 3H, –OCH₃), 4.78 (dd, *J* = 7.6 Hz, *J* = 6.4 Hz, 1H, –CH=), 6.90–6.95 [m, 2H, Ar-H(–5, –6)], and 7.06 ppm [d, 1H, Ar-H(–2)]. HRMS (ESI): *m/z* = [M–H][–], 197.0810 (calcd mass: 198.0892, formula: C₁₀H₁₄O₄)

BGP synthesis from GPD

Water (15 mL) and GPD (0.4 g, 2.0 mmol) were placed in a 50-mL round-bottomed flask; the GPD was dissolved at room tempera-

ture. Methanesulfonic acid (44 mg, 0.46 mmol) was added; the mixture was allowed to react for 22.5 h at 65 °C. Aqueous NaOH (1 N, 0.46 g) was added to neutralize the acid catalyst, methanesulfonic acid, and then the target product was extracted twice (10 and 5 mL) with ethyl acetate to provide 0.32 g of a viscous liquid. The HPLC purity of the liquid, 3,3-bis(4-hydroxy-3-methoxyphenyl)-1-propanol, was 68% (determined by area normalization without sensitivity correction). This liquid was chromatographed on a silica-gel column (Wakogel C-200) with ethyl acetate:toluene (2:3) to provide 0.15 g of purified product (yield: 49%). The NMR spectra (Figure S2a,b in the Supporting Information), HRMS, and IR of synthetic BGP were as follows.

¹H-NMR (CDCl₃): δ = 1.34 (m, H, –OH(–CH₂–OH)), 2.23 (ddd, *J* = 8.0 Hz, *J* = 6.4 Hz, *J* = 6.4 Hz, 2H, –CH₂–), 3.61 (m, 2H, –CH₂–O–), 3.82 (s, 6H, –OCH₃), 3.98 (t, *J* = 8.0 Hz, 1H, –CH=), 5.53 (s, 2H, Phenol–OH), 6.70 [d, *J* = 2.0 Hz, 2H, Ar-H(–2)], 6.76 [dd, *J* = 8.4 Hz, *J* = 2.0 Hz, 2H, Ar-H(–6)], and 6.84 ppm [d, *J* = 8.4 Hz, 2H, Ar-H(–5)].
¹³C-NMR (CDCl₃): δ = 38.76 (–CH₂–), 46.75 (–CH=), 55.92 (–OCH₃), 61.23 (–CH₂O–), 110.65, 114.29, 120.20 (ArC protonated), 136.82, 144.11, and 146.58 ppm (ArC substituted). HRMS (ESI): *m/z* = [M–H][–], 303.1227 (calcd mass: 304.1311, formula: C₁₇H₂₀O₅). IR: $\tilde{\nu}$ = 3530, 3451, 3139, 3010, 2962, 2936, 2894, 2842, 1604, 1514, 1275, 1034, and 817 cm^{–1}.

Coniferyl alcohol synthesis from GPD

GPD (164 mg, 0.83 mmol), isopropanol (2 g, 33.3 mmol), and triethylamine (0.4 g, 4.0 mmol) were placed in a 10 mL test tube and stirred for 48 h at 85–90 °C, after which the reaction mixture was transferred to a 50 mL round-bottomed flask and 20 g of water was added. Volatiles were removed by reduced-pressure distillation in a 45 °C bath until the reaction mixture weighed 7 g, after which 60 mg of 10% HCl was added. The reaction mixture turned cloudy at pH 5. The mixture was transferred to a separatory funnel and extracted twice with 2 mL of ethyl acetate. Evaporation of the ethyl acetate provided a viscous liquid product, coniferyl alcohol (118 mg, 79%; HPLC purity, 90%). The crude coniferyl alcohol (118 mg) was chromatographed on a glass column (inner diameter: 23 mm, packed length: 100 mm) packed with silica gel (Wakogel C-200) using a 2:3 ratio of ethyl acetate:toluene. The purified coniferyl alcohol (83 mg) was obtained as white needle crystals (HPLC purity: 97%, melting point: 72–74 °C).

GVK synthesis from GHP

GHP (2.0 g, 10.2 mmol) was dissolved in 24 g of concentrated hydrochloric acid (36% HCl) in a 100 mL Erlenmeyer flask, after which the reaction mixture was placed in an oil bath and the reaction was performed for 0.5 h at 90 °C. The flask was cooled to room temperature. The resulting crystals were filtered off, washed with water, and dried in vacuo to yield 2.1 g of 3-chloro-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (2.1 g, 96%). The HPLC purity was 96% and the impurity included unreacted GHP.

3-Chloro-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (1.41 g, 6.5 mmol) was dissolved in a 100 mL Erlenmeyer flask containing 20 mL of ethanol at room temperature, after which sodium ethoxide (0.9 g, 13.2 mmol) was added at room temperature. After 1.5 h, the reaction mixture was neutralized by addition of 10 mL of water and 2.4 g of 10% HCl. Removal of ethanol by reduced-pressure distillation resulted in the separation of a brown, oily substance, which was extracted with 10 mL of ethyl acetate and washed with water. The ethyl acetate was removed by reduced-pressure distillation, forming 1.22 g of crude GVK with a HPLC purity of 93%. The

crude GVK was chromatographed on silica gel (Wakogel C-200) using toluene. The purified product yield was 61% and its HPLC purity was 99%. The ¹H-NMR spectrum (Figure S3 in the Supporting Information) and HRMS were as follows.

¹H-NMR ([D₆]acetone): δ = 3.93 (s, 3 H, -OCH₃), 5.85 (dd, *J* = 10.4 Hz, *J* = 2.0 Hz, 1 H, C=CH₂), 6.36 (dd, *J* = 16.8 Hz, *J* = 2.0 Hz, 1 H, C=CH₂), 6.95 [d, *J* = 8.0 Hz, 1 H, Ar-H(-5)], 7.39 (dd, *J* = 16.8 Hz, *J* = 10.4 Hz, 1 H, -CH=), 7.61 [d, *J* = 2.0 Hz, 1 H, Ar-H(-2)], 7.65 ppm [dd, *J* = 8.4 Hz, *J* = 2.0 Hz, 1 H, Ar-H(-6)]. HRMS (ESI): *m/z* = [M-H]⁺, 177.0550 (calcd mass: 178.0630, formula: C₁₀H₁₀O₃)

Radical polymerization of GVK

Crude GVK (160 mg) obtained above, 1 g of tetrahydrofuran dried for 2 days over a 3 Å molecular sieve, and 7 mg of 2,2'-azobisisobutyronitrile as a radical initiator were placed in a 10-mL test tube, sealed with a silicone rubber stopper, and purged with nitrogen, after which the tube was placed in a silicone oil bath at 60 °C and left to react for 45 h. The solvent was removed by reduced-pressure distillation to yield a pale-yellow powder (151 mg). The polymerization product (38 mg) was dissolved in 5 g water; 8 mg of aqueous 1 M NaOH was added with agitation at room temperature. HCl (10%) was added to lower the pH to approximately 6; the deposited precipitant was filtered and washed with water. Drying in a desiccator formed a 38 mg solid. The NMR spectra of the compound are shown in Figure S4a,b in the Supporting Information.

Acknowledgements

This research was funded in part by the Funding Program for Next Generation World-Leading Researchers (number GS031) of the Japan Society for the Promotion of Science (JSPS) awarded to Y.O., a JSPS KAKENHI Grant (numbers 15K00649) awarded to Y.O., a research grant for Exploratory Research on Sustainable Humanosphere Science from Research Institute for Sustainable Humanosphere (RISH), Kyoto University and the Advanced Low Carbon Technology Research and Development Program (ALCA) of the Japan Science and Technology Agency (JST) awarded to Y.O. and H.N. We thank Mr. Makoto Roppongi (University of Utsunomiya) for technical assistance.

Keywords: biomass • enzymes • lignin • molecular recognition • reaction mechanisms

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Manuscript received: September 6, 2016

Revised: October 31, 2016

Accepted Article published: November 22, 2016

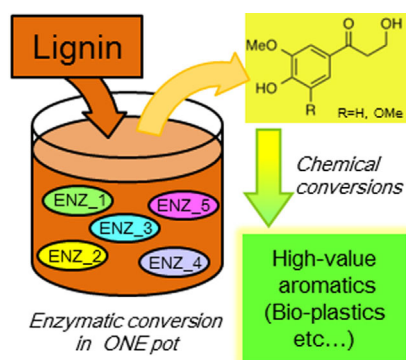
Final Article published: ■ ■ ■ 0000

FULL PAPERS

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Enzymatic Specific Production and
Chemical Functionalization of
Phenylpropanone Platform Monomers
from Lignin



Enzymatic cleavage of lignin: Marine bacterial enzymes recognize and cleave various lignin substructures near β -O-4 linkages and produce two phenylpropanones selectively. These monomers are promising platforms for producing bio-plastics and other functional chemicals in medical and cosmetic industries. This study provides a new stream for “white biotechnology”.